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North Carolina University at Chapel Hill

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May 1976

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VIRION AGGREGATION AND DISINFECTION OF WATER VIRUSES BY BROMINE

by

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Grant No. R802946

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A dynamic system, in which disinfectant exposure time intervals as low as 0.5 seconds was developed. Polio and reo virus preparations, containing essentially all single particles, as indicated by electron microscopy, were tested in this system. The results indicated that reovirus single particle suspensions were 30 times as sensitive to bromine as poliovirus single particle suspensions, both showing essentially first order reaction kinetics with disinfection rates of 3 $\log_{10}$ per second and 6 $\log_{10}$ units per minute, respectively. Inactivation rates with slightly aggregated reovirus deviated from first order kinetics, the reaction rate declining continuously in a manner consistent with the degree of aggregation shown by electron micrographs. In other experiments, aggregated reovirus showed some survival after 4 minutes exposure to the same bromine concentration that reduced single particle preparations below that level in 1.33 seconds. Thus aggregated reovirus was nearly 200 times as resistant as dispersed reovirus and substantially more resistant than dispersed poliovirus. Temperature effects on disinfection rates using dispersed poliovirus were also investigated. The report demonstrates the effects of virus aggregation on disinfection kinetics and provides the basis for comparing the resistance of different viruses to different disinfectants under various conditions.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This report describes new techniques for studying virus inactivation kinetics and provides new information on the effects of virus aggregation on the inactivation of enteroviruses in water by halogens. This information is essential in assessing the adequacy of water treatment technology for production of virologically safe drinking water.

Francis T. Mayo, Director
Municipal Environmental Research
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ABSTRACT

Water suspensions of polio and reo virus have been prepared containing essentially all single particles as shown by quantitative electron microscopy. Disinfection of such preparations by bromine was so rapid that special apparatus had to be designed and built to deal with exposure times as short as ½ second. The infectivity of this poliovirus declined at the rate of 6 Log_{10} units per minute while reovirus lost 3 Log_{10} units per second. The reactions with both viruses were essentially first order, giving approximately straight lines on semi-log paper. Even slightly aggregated reovirus produced a curve, the reaction rate declining continuously, in a manner consistent with the degree of virion aggregation seen in the electron micrographs. It appears from these experiments that small aggregates of bromine-treated reovirus may owe their superior plaque forming potency to complementation.

Disinfection of water containing single polio virions by 10 μM bromine was approximately 2 times more rapid at $10^{o}C$ than at $2^{o}C$ and again 2 times more rapid at $20^{o}C$. The rate was approximately proportional to bromine concentration at 10^{o} and 20^{o} but at 2^{o} there was some evidence of non-linearity at bromine concentrations less than 1.0 μM, suggesting a difference in mechanism of inactivation.

Although reovirus single virion suspensions are 30 X more sensitive to bromine than poliovirus, some preparations of aggregated reovirus have survived 4 minutes at the 10^{-4} level with the same bromine treatment that reduced single reo virions below that level in 1-1/3 seconds. Thus the aggregated reovirus is almost 200 times more resistant than dispersed reovirus and, thus, substantially more resistant than single poliovirus particles.

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INTRODUCTION

There have been many papers written about the survival of virus during exposure to disinfecting agents and most of them include, in the discussion of their results, some speculation regarding the possible influence of virus particle aggregation. This paper differs from the others. It provides direct evidence of the kind and degree of aggregation that was present among the virions that were treated with bromine as well as the PFU titers of the starters and the survivors. This physical evidence comes from electron microscopy (EM) of virus samples prepared by methods especially contrived to avoid the aggregation artifacts which are inherent in ordinary preparative techniques that have been designed primarily to show ultrastructure. Two methods for doing this have been devised here. Both of them are based upon the premise that the virus particles and clumps must become attached to the surface for observation while the surface is in contact with the whole body of the water suspension; they must not be deposited there during the time the water suspension of virus particles is drying because the attendant forces of surface tension exert strong aggregation effects on suspended particles. The first of these techniques which works well with the larger viruses, including reovirus, involves sedimentation of the particles by centrifugation onto an agar receiving surface from which they can be transferred by pseudoreplica to the electron microscope. The other involves kinetic attachment of the particles by Brownian bombardment of an aluminum-coated collodion film which after thorough washing, can be examined directly in the electron microscope. This method gives count and aggregation data on small viruses such as polio which do not yield to the sedimentation pseudoreplica method.

Observations on the physical state of aggregation of a virus in water requires particle concentrations great enough so that EM pictures will show enough particles and groups of various sizes so that their numbers can be established with the necessary statistical significance. Also, they must be sufficiently purified so that there is no doubt about recognition of the particles, as virus, and distinguishing the separate individuals in the groups. This means about 10^8 per ml for reovirus (by sedimentation count) and about 10^{10} per ml for polio by the kinetic attachment (KA) method. Such concentrations may seem very high but actually the corresponding PFU titers are about 2×10^6 per ml for reo and 10^8 per ml for polio, both of which are well within the range normally experienced in unconcentrated lysates of infected cell cultures. The difficult problem is encountered in storage of the virus for aggregation experiments.

In our experience small volumes of purified virus suspensions are exceedingly unstable with regard to physical state. This makes it very difficult to transport them (by mail, etc.) for count or further experimentation, or to store for repeated experiment at a later date. Fortunately we have learned one way to do it and the means is a consequence

of our method of purification. When either polio or reo virus is banded in a sucrose gradient and recovered in sucrose concentration about 20-25% and stored unfrozen in an ordinary refrigerator, the state of dispersion is much more stable than it is under any other conditions that we know. There has been no bacterial growth in periods up to 6 months and polio virus seems not to aggregate at all under these conditions. Reo virus does aggregate but very slowly.

CHAPTER I

The Initial Fast Reaction of Bromine (HOBr) with Reovirus in Turbulent Flowing Water

In a previous publication (1) we have shown that the inactivation of reovirus by bromine in water is characterized by an initial rapid phase followed by an extended period of much greater resistance in which infectivity persisted, usually at about the 10^{-4} level of survival. Before examining in further detail the behavior of the resistant fraction of the plaque forming units which appeared to be a few large aggregates, it seemed wise to determine the kinetic features of the initial fast reaction. Here mostly single virions and associated small aggregates (groups of 1 to 10 virions) are likely to be involved and from them it should be possible to determine the basic inactivation rate for the virus. However, the reaction in this region proceeds so fast that the survival ratio is reduced 2 to 4 $\log_{10}$ units within the first minute at pH 7, and 2°C even at bromine concentrations as low as 3 to 5 μM. It is clear that the exposure of virus to bromine solutions in stirred beakers cannot yield satisfactory kinetic data for reaction times of the order of ½ to 20 seconds. Consequently a different experimental approach was designed. Forty-three reaction rate experiments have now been done in this way and we believe that the results of the last few of these experiments that are presented here will justify a detailed description of the method and apparatus. They also establish the inactivation rate for single reo virions and indicate, to some extent, the effect of small-number aggregates on survival of infectivity.

Materials and Methods (General)

Virus, Culture and Plaquing. Reovirus type 3 (Dearing strain) was grown in and all plaque titrations were made on monolayer cultures of L cells as previously described (1), with the following exceptions: Cells were maintained in 200 ml milk dilution bottles and passaged into 32 oz. prescription bottles for growth of virus. Virus was harvested at 16 to 18 hrs after infection at 10-20 pfu/cell rather than 20 to 24 hours as previously noted (1). In addition, the virus was extracted fnom the cells in 6 ml of PBS without calcium and magnesium and 4 ml of Freon 113 for 2 min. at one-half speed in a Sorvall Omni-Mixer. The virus was placed on 20 → 40% w/w sucrose gradients in 0.05 M phosphate buffer at pH 7.2, and centrifuged at 25,000 RPM in the Beckman SW 27 rotor at 4°C for 1 hour. The virus, collected from the lower of two bands seen with a collimated beam of light, was allowed to remain in the sucrose at 4-6°C, and was not pelleted. The virus stored in this manner retains its infectivity for several weeks and the state of physical aggregation is quite stable. There is no bacterial or fungal growth and

the sucrose does not produce any detectable bromine demand under the conditions of our experiments. Immediately prior to use the virus preparation was diluted 1:10, hence the sucrose level as used in an experiment was around 3%.

Plaque titrations were performed in tightly stoppered 1 oz. prescription bottles under an overlay of 1% Difco agar containing Medium 199, 5% Fetal Calf Serum, 0.245% $NaHCO_3$, and 0.003% Neutral red. Plaques were counted after 6 days at 37°C.

Physical Assay of Virus and Virion Aggregation. Single virus particles and aggregates were counted by electron microscopy using the agar pseudoreplica method (1).

Zonal centrifugation. Resolution of both crude and purified virus particle populations into sedimentation velocity spectra (2) was done with a BXIV titanium zonal rotor provided by Dr. Norman Anderson (3) of the Oak Ridge National Laboratories operating in a Sorvall OTD2, fluid-drive ultracentrifuge.

Bromine, Glassware and Water. Bromine measurement and preparation of glass apparatus and demand-free water were the same as previously reported (1) except that amperometric titrations were employed together with the method of Taylor (4) and all phosphate buffers were made with Fisher primary standard potassium salts.

The Kinetic Experiment

Object

The apparatus must provide a large enough volume of water to insure stability of temperature, bromine concentration and flow rate through the reaction siphon tube (Fig. 1) for a period of several minutes. The reaction tube is 3/8 inch inside diameter and the bottle (20 liter) height is adjustable so that turbulent flow at the desired rate can be achieved. Turbulence is essential, so the Reynolds Number has been kept equal to or greater than 3000 in all experiments. The reaction tube has 5 access ports covered by disposable serum bottle stoppers. Five 5 ml syringes with needles are inserted. The first of these (at the left) carries the virus inoculum (5 ml) which, when triggered, starts the weight-driven plunger which delivers the inoculum steadily for 5 seconds, into the turbulent stream of buffered bromine water. There is a 6 mm diameter disk located on the axis of the tube just downstream of the inoculum needle tip, to aid in mixing.

The time of transit from injection point to sampling point will be determined only by the flow rate and the distances D_1, D_2, etc. It is not in any way dependent on when the sample is taken as long as the sample is taken within the 5 second interval during which the "polluted" water passes the sampling port in question.

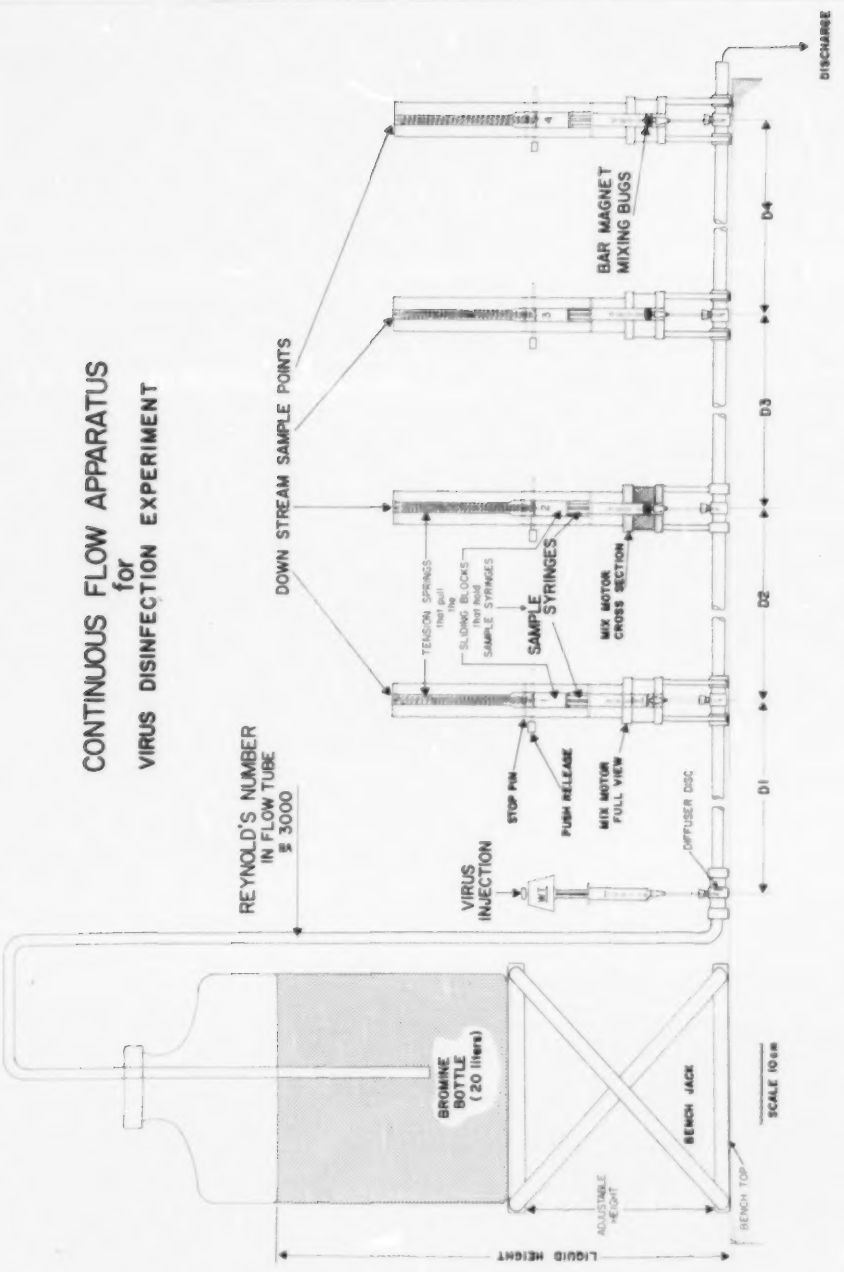

Fig. 1

Apparatus for injection into and recovery of virus from a turbulent stream of water containing a disinfecting agent such as bromine.

Each of the 4 sampling syringes contains 1 ml of 2 mM sodium thiosulphate solution, 1 ml of air and a small Teflon-coated magnetic stirring bar. The syringe plungers are held in metal blocks attached to helical springs, each under tension and restrained by a release pin that can be pushed at the desired time. When the spring is released the syringe draws exactly 1 ml from the flowing stream. This is mixed with the thiosulphate solution to stop the reaction of the bromine on the virus. For virus-bromine exposure times of 1 second or less additional precautions were taken to insure instant mixing. This is done by means of the magnetic stirring bar which is made to rotate at 1800 RPM by 2-pole AC motor windings surrounding each sampling syringe. These are stator windings used in small electric servo motors kindly provided for us by the Sperry Rand Company of Durham, N. C.

Operation

Twenty liters of buffered chlorine-demand-free water is prepared and adjusted to the temperature required for the experiment. Enough bromine is added 17 hours before the experiment to bring the concentration in the bottle to approximately the right value. Just before the experiment, more bromine is added to adjust the concentration to exactly the required level. Previous experiment has established the exact level of liquid in the bottle to produce the desired flow rate (range 20 to 40 ml/sec. in different experiments). This level is marked for easy reference and all experiments start at this level. Beginning with the liquid at a higher level, the siphon is started and the flow continued for a few minutes to allow the tubing to adjust to the temperature in the bottle and when the liquid level reaches the marked starting level the virus injection is started. The stirring bars in all four sampling syringes are already running in their 1 ml volumes of thiosulphate solution and it only remains to trigger each of the syringes as the flood passes and also catch a sample of the virus-bromine mixture at the discharge end to determine its bromine concentration for comparison with that in the bottle. Neutralized bromine-virus mixtures from the sampling syringes and the virus from the inoculation syringe are then assayed for virus by the plaque method.

The dilution of virus, as it enters the flowing stream is just the ratio of the rates of injection and stream flow. The injection syringe is timed by direct observation and stopwatch. The stream flow rate is easily obtained by timing the outflow of some conveniently measured volume such as 500 ml. Variation in flow rate with liquid level in the bottle is negligible during the experiment time, which has not been over 20 seconds, because of the large (approx. 700 Cm^2) surface area.

Tygon tubing is convenient for the flowing stream but we have found that it absorbs substantial quantities of bromine. Glass is difficult to manage without catastrophic accidents. However, "High Density" polyethylene tubing has performed well. It is readily cut to desired lengths and it exerts negligible bromine demand.

Experiments and Results

Several experiments were made at 10°C with samples taken at 4 second intervals. These were made with virus prepared in the same way as that of our earlier publication (1) which involved pelleting of the purified virus to remove sucrose. These were done at 3, 5.8 and 5.9 μM bromine concentrations (Fig. 2) and they all show approximately the same level of persistant resistance previously reported in exposures of longer duration. They show also that the initial fast reaction is taking place during the first 4 second interval. At this point we added the magnetic mixing devices to the sampling syringes and reduced the length of the flow tube to give samples at one and later at 1/2 second intervals. The temperature was reduced also, from 10° to 2°C to reduce the reaction rate and still permit the use of bromine concentrations high enough to hold constant when confronted with expected bromine demand of the virus.

Three experiments were made at 2.8-3.0 μM bromine concentration with virus prepared without pelleting. Two were done with 1 second time intervals and one with the intervals reduced to 1/2 second. The frequency of aggregates observed by electron microscopy in this virus preparation is shown in the graph, Fig. 3. Of the whole particle population, 73% are singles and the groups appear to be distributed in such a way that the log frequency of each group size is a linear function of the log of the number in that group. We (5) and others (6) have observed this same distribution with other viruses and also with polystyrene and acryl particles of comparable size. The inactivation of the virus by bromine in these three experiments is shown in the semilog plot of survival ratio vs time in Figure 4. It is curved all the way. A single line has been drawn by inspection through all three sets of points. Correlation of this result with the aggregation distribution (Fig. 3) will be made later, in the discussion.

Zonal Centrifuge Experiment #1

An effort was made to obtain a preparation of virus containing only single particles. This is needed to provide a frame of reference for comparison with the above results with aggregated virus. Fifteen ml of a purified virus preparation, aggregated as shown in Fig. 5 was layered over a 15-30% w/w sucrose gradient with the BXIV zonal centrifuge rotor running at 2000 RPM. The total number of virions put into the rotor was 10^{11} by EM count. After operating at 25,000 RPM for 30 minutes ($\Sigma \omega^2 t = 1.44 \times 10^{10}$) at 20°C, the 640 ml volume of the rotor was completely displaced by 35% sucrose piston fluid and 25 ml fractions were collected in 1 oz. bottles. The virus particle count on each was made by EM after dilution with 0.85% filtered sodium chloride solution. A dilution of at least 1/10 is required otherwise the agar block that receives the sedimented virions in the pseudoreplica counting process will float to the surface because of residual sucrose. Particle counts are plotted against fraction numbers and a scale of radial distances is included on Fig. 6. Densities in the sucrose gradient were measured by direct pyknometer weighings.

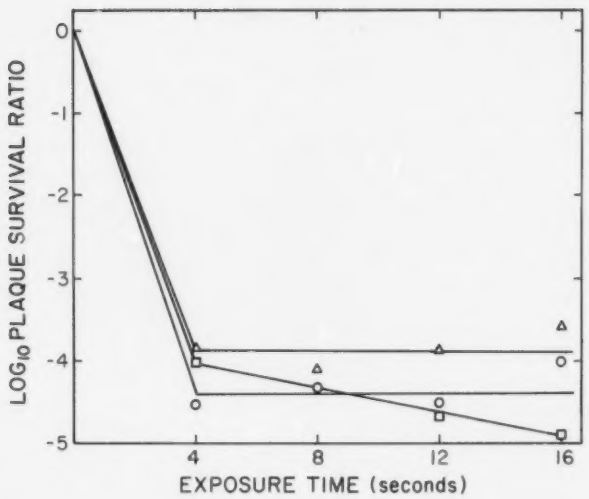

Fig. 2

Survival of reovirus infectivity in water containing 3 μM (triangles), 5.8 μM (circles) and 5.9 μM (squares) bromine at 2°C and pH 7.0. The rapid phase of the reaction is done in less than 4 seconds and the slower phase has been shown to be caused by a few large aggregates.

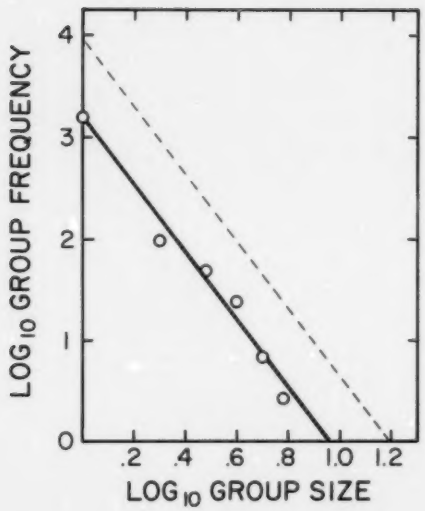

Fig. 3

Frequency distribution of aggregates observed by electron microscopy (circles). The dotted line indicates the same distribution for a more concentrated suspension containing a total of 10,000 particles.

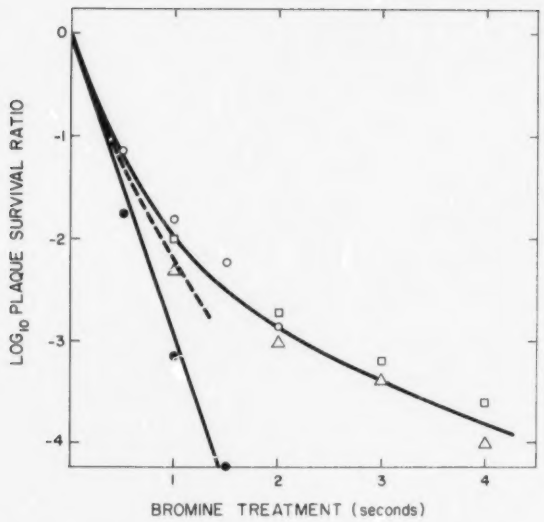

Fig. 4

Inactivation of reovirus during the first 4 seconds of contact with 2.9 μM bromine in water at pH 7 and 2°C. Three separate experiments are shown with different symbols. The frequency of aggregates of different sizes is that shown in Fig. 3. Filled circles show near linear response of a preparation containing nearly all single virions. The broken line is a prediction for the survival of the same kind of particles as those of the straight line but aggregated as shown in Fig. 3 and subjected to degradation according to the multiple target concept as calculated by the Poisson limit.

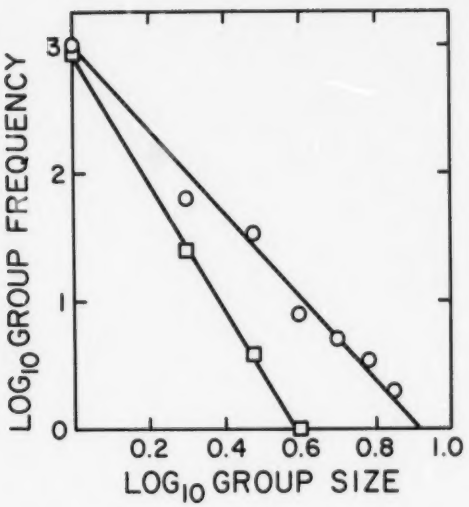

Fig. 5

Frequency distribution of aggregates observed in starting virus preparation for zonal centrifuge experiment #1 (circles). The squares show how the distribution was altered by removal of most of the aggregates from the region of the principle peak (Fig. 6).

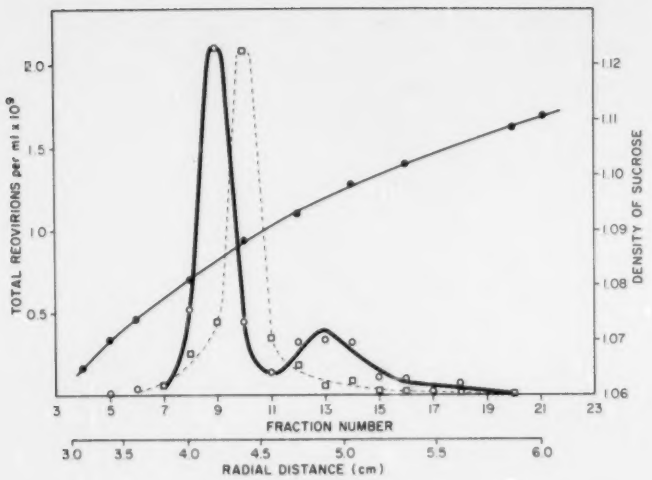

Fig. 6

Sedimentation velocity spectrum obtained in the zonal centrifuge of spontaneously aggregated reovirus (solid line). Ordinates are particle counts made by electron microscopy. Sixty nine percent of the virus is in the major peak which has 93% single particles and a few remaining small aggregates as shown in Fig. 5. The secondary peak contained mostly pairs and triplets in Fraction 13. The dotted line shows a similar spectrum of crude (unpurified) reovirus showing that the original state of freeze-thaw-released virus contains very few small aggregates. Large aggregates have sedimented to the rotor rim.

Fractions 4 through 10 contained 69% of the recovered virus. In the peak fraction (#9) there were 93% single particles. Six percent were in pairs and the remaining one percent were triplets and groups of four; see bottom line of the graph Fig. 6. Thirtyone percent of the total recovered virus made a broad secondary peak with maximum count in bottle # 13 in which 63% of the particles it contained were in pairs. The total count of all the fractions indicated 110% of the 10^{11} particles that were put in. Apparently there was no detectable loss in the partition process and calculations from the EM frequency distribution chart (upper line of Fig. 6) predict that 71% of the in-going propulation was single particles, a figure in excellent agreement with that observed in this actual sedimentation velocity spectrum from the zonal centrifuge.

Zonal Centrifuge Experiment #2

Inasmuch as zonal selection provides a method of obtaining very high percentage single reovirus particle suspensions we prepared a fresh quantity of purified virus containing about 50 x more virus than that of the above zonal fractionation. This preparation yielded the velocity spectrum with singles peak like that of Fig. 5 but the fractions 12 through 19 that contained aggregates of 2 to 8 particles constituting 31% of the first population examined now contained aggregates the same size range but relatively few (less than 10% of the total). Apparently freshly prepared Freon-extracted virus has very few aggregated particles. In the peak fraction (#10) there were 85% singles. It is possible that at this high concentration (2.5 x 10^{10} virions/ml) some spontaneous aggregation took place before preparations could be made for electron microscopy. A part of this fraction was frozen immediately in order to preserve both infectivity and physical dispersion for subsequent bromine inactivation experiments.

Zonal Centrifuge Experiment #3

One more zonal velocity spectrum experiment was made with crude freeze-thaw lysate of infected L cells without even low speed clarification. This crude preparation contained the virus from the same number of infected cells as that providing virus for the last experiment just described above. Again a single particle peak was observed (Fig. 5) and again there was very little virus in the region where aggregates of 2 to 8 were seen in substantial numbers in the first experiment. This unexpected result drew attention to the fact that the total quantity of virus recovered from the crude starting material was only about 1/5 of that usually recovered by Freon extraction of an equal number of infected cells. It would appear that an excellent fraction of single particles can be obtained in this way with no previous purification, that there are very few small aggregates in such a preparation but that the major part of the virus must have been in large aggregates or combined with larger cell debris which sedimented beyond the sampling range and reached the rim of the zonal rotor.

Bromine Inactivation of "Singles" fraction from velocity spectrum:

The singles peak fraction from zonal velocity spectrum (ZR2 above) was frozen at -40°C in 1 ml vials to preserve infectivity and state of dispersion. Subsequently thawed samples had not lost infectivity and the relative numbers of single particles and groups in the size range of 2 to 8 had not changed but bromine inactivation showed that changes had nevertheless taken place. After a rapid start, the reaction became slower and appeared to reach a resistant level at about 10^{-3} survival after 2 seconds at 3 μM and 2°C. If these surviving PFU are aggregates, they must have "grown" from single particles during the short time interval before freezing or in the thawing process.

Five ml of the same virus was treated with bromine in the same way except that it was centrifuged 17 minutes at 20,000 RPM at 20°C in a Beckman SW50 rotor. Only the top 4 ml was used in the bromine experiment. All large aggregates should have been removed from this preparation. The resulting rapid linear decline in PFU (Fig. 4) provides excellent confirmation.

Partition of crude Reovirus between singles and large aggregates by means of a sucrose gradient in the swinging bucket centrifuge rotor.

Zonal sedimentation velocity spectra of crude reovirus (ZR3 above) yields a prominent sharp band of single virions and relatively few small aggregates but most of the virus seems to have sedimented much faster than either. An experiment was done in the large (37 ml) buckets of the Beckman SW-27 centrifuge rotor to determine if possible the quantity and physical state of virions and the plaque titer of the two major components of this particle population, the singles band and the pelleted material. In order to do this a 15-30% sucrose gradient was established and 10 ml of the freeze-thaw lysate (the same as that used in experiment ZR3) was layered over it. After spinning at 25,000 RPM (mean centrifugal field 81,000 XG) for one hour, the supernatant fluid above the visible singles band was discarded. Ten ml including the singles band was collected then the pelleted material was resuspended in the remainder of the supernatant fluid by pumping with a pipette. These two fractions will be called Band 1 and Pellet 1.

An equal quantity of the crude virus preparation was extracted with freon and layered over a similar sucrose gradient, centrifuged and harvested in the same manner, Band 2 and Pellet 2.

Half of the resuspended Pellet 1 material was extracted with freon, made up to the same volume (10 ml) and banded and harvested like the previous ones, Band 3 and Pellet 3.

A part of both fractions B1 and P1 were treated 30 seconds with 20 KHz acoustic waves from the micro tip of a Branson Sonofier Model LS-75. Sample volumes treated were 5 ml each and they were immersed

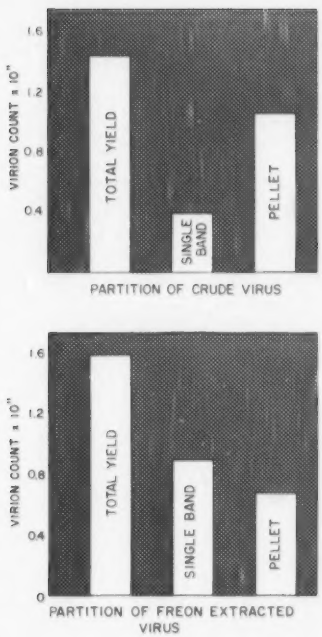

Fig. 7

The total yield of reovirus extracted from a given number of infected cells is about the same whether it is done by 3 freeze-thaw cycles (top panel) or by freon extraction (bottom panel) but only 27% of it appears as a band of single particles when the crude virus is sedimented in a 15-30% w/w sucrose gradient. The rest is in the pellet. For freon-extracted virus the partition is 53% to 47%. Fig. 6 shows that there are very few small aggregates present.

in ice water during treatment. The maximum temperature attained was 15°C. There were 8 samples in all prepared for virion count and observation of aggregation in the electron microscope and for plaque titration. The results of physical assay are shown in Fig. 7. Particle count of pelleted fractions from Freon-extracted preparations contained a large amount of cellular debris which made the virions difficult to count. Nevertheless the total particle yield (sum of counts from pellet and band) was approximately the same (Fig. 7). Freon extraction yielded 57% of the particles in the band but with the crude virus only 28% was in the band. Freon extraction of virus from the pellet fraction of the crude virus experiment (P1) yielded 67% of the particles in the band. Apparently the freon extraction process is quite efficient for extraction of reo virus in monodisperse form from the infected cells.

Plaque titration of banded and pelleted fractions from several identical partition experiments have shown 1/7 of the freon-extracted virus to be in the pellet and 6/7 in the band. The partition of PFU in crude preparations has been erratic. Always the pellet fraction has much more than the band and once there were 50 X as many PFU in the pellet as there were in the band. Treatment of banded and pelleted fractions with 20 KHz acoustic waves have not made any substantial change in particle count but in this case it reduced the plaque titer of the pelleted fraction by a factor of 10. The same treatment made no change in the titer of the well-dispersed virus in the banded fraction.

Discussion

Reovirus was chosen for this work from among the many water viruses because of its size which makes it readily amenable to physical assay by two essentially independent methods. Earlier work has shown (1) that the reo particle counts and aggregation analyses made by the Kinetic Attachment method of preparation for electron microscopy yield the same results with suspensions of 10^{10} virions per ml. as those obtained by the sedimentation or agar pseudoreplica method on the same suspension after approximately 100 fold dilution. Demonstration of this fact is essential, of course, for if it were not so, the dilution of suspensions of aggregated virus for plaque assay would yield no information about the bromine resistance or plaquing efficency of the aggregates seen in the electron microscope. For this reason reovirus may provide a useful model of reference when experiments of this kind are made with the many smaller picorna viruses that are of greater significance as water pollutants but which are more difficult to assay by physical means.

The reaction of bromine (3 μM) with preparations of reovirus in water at pH 7 is shown here to destroy 99.9% of the PFU in one second at 2°C if the preparation contains predominately single virions. After the most strenuous efforts to remove all aggregated virus, the logarithm

of the survival ratio is a linear function of the time of exposure to the bromine at least to the level of 1:10,000 surviving PFU, the titer falling to the rate of 3.0 $\log_{10}$ per second. This is 25 x faster than the rate observed with poliovirus under similar conditions of bromine exposure (7). It is more than 100 times faster than rates indicated by Scarpino et al (8) and Weidenkopf (9) respectively for chlorine at pH 6 and the same molar concentration and approximately the same temperature. Comparison of bromine rates at pH 7 with those of chlorine at pH 6 seems most appropriate for comparison of the effects of HOCl and HOBr.

While reovirus, velocity banded in a sucrose gradient is usually 90 to 95% single particles, it gradually aggregates in such a way that the log of the frequency of any group size becomes and remains a linear function of the log of the number of particles in the group (Fig. 3). Inactivation by bromine of such aggregated virus is characterized by a continuously decreasing decay constant. It would appear from this that plaque-forming aggregates are more resistant to bromine than single virions.

If the probability of survival of a single virion after a mean dose m of bromine is taken to be e^{-m}, and the survival of one of the particles of an adhering pair as $(1+m)e^{-m}$ etc. and for a group of i particles, $\left(1+m+\frac{m^2}{2}+\cdots\cdots\frac{m^{(i-1)}}{(i-1)!}\right)e^{-m}$ then the titer of a mixture of N1 singles + N2 paris + N3 triplets etc., would be given by:

$$T = N_1 e^{-m} + N_2(1+m)e^{-m} + N_3\left(1+m+\frac{m^2}{2!}\right)e^{-m}$$

in which the coefficients N_1, N_2, N_3 etc. can be supplied by the EM pictures (Fig. 3). Now the unit dose m can be taken from the experimental inactivation of an essentially singles population (Fig. 4). Thus we can calculate and plot the expected survival curve for this particular mixture of aggregates. The result is the dotted line of Fig. 4 which has the approximate shape of the observed curve but not quite enough departure from the straight line to match the experimental curve. Inherent in the above analysis is the assumption 1) that inactivation of a virion is the result of a single vital event which either does or does not occur during the time interval specified, 2) that survivors are undamaged, and 3) that the other virions in a clump containing one survivor do not help or hinder its chances of making a plaque. The authors are doubtful that all of these assumptions are valid, particularly the last one and they point out also that before bromine treatment there are approximately 100 x as many virions as there are PFU in reo virus preparations. This means that none of the group sizes from 2 to 10 would have over a 10% probability of containing more than one plaque forming particle before the inactivation began. It appears that some degree of complementation may be involved in survivor plaque formation by aggregates to account for the observed curve.

Preparations of reovirus containing 90 to 95% single particles can be obtained easily from either freon-extracted lysates of infected L cells or directly from crude lysates in which virus has been released from the cells by 3 cycles of freezing and thawing. The freon extracts yield about 2 x as much virus but both appear equally well purified when harvested from the sucrose gradient in the singles band in either the swinging bucket or the zonal centrifuge rotor. Slow spontaneous aggregation occurs in preparations of reovirus stored at 4-6°C and in these mixtures of aggregates there is a continuum of sizes the logarithm of whose frequency is a linear function of the logarithm of the number of virions in the group. As aggregation continues, the slope of this line changes but the line remains straight. These aggregates appear to contain nothing but virions (like those in the experiment of Fig. 4). They are "pure" aggregates. But the major part of the virus released from infected cells by freezing and thawing alone, without homogenization with freon, must consist of aggregates of a different kind. One difference is immediately seen in the sedimentation velocity spectrum of such crude virus which is produced in the zonal centrifuge rotor. While about 1/5 of the total virions and 1/50 of the PFU appear in a prominent "singles" band there are very few groups of 2 to 10 such as those seen in spontaneously aggregated preparations (Fig. 6). The supporting experiment in the swinging bucket rotor supplied the quantitative data on the partition of the virus in the crude preparations. It indicates clearly that the fast-reaction kinetics established in this report, and doubtless required for understanding of the bromine-virus reaction, have been obtained with about 1/5 of the virions normally present in crude extracts of infected cells, while the remaining 4/5 is in quite a different physical state, a state that appears to be much more resistant to bromine action. One can reasonably expect that the virus found in polluted water will be in a state more like the crude than the purified preparations employed in this work. So it will be necessary to examine in some detail the nature of the natural occurring aggregates as well as the spontaneous or "pure" aggregates and their resistance to halogen ions if conditions in polluted water are to be understood. The data from the initial fast reaction with single virions and small groups provides a step in this direction.

CHAPTER II

Bromine Inactivation of Single Poliovirus Particles in Water

The kinetic features of the reaction of disinfecting agents on viruses in water have usually revealed a significant departure from the simple straight line expected from a reaction of the first order. Several authors have suspected (10,11,12,13) that aggregation among the virions is responsible for some if not all of this irregularity. And this seems very likely because several enteric virus, including polio, are generated in large tightly packed cytoplasmic crystals (14) whose release into water suspension is not likely to be accompanied by complete dispersion. Then too, laboratory preparation of virus suspensions for halogen inactivation experiments have often involved concentration by pelleting in the ultracentrifuge, a process that can induce aggregation even among previously well-dispersed particles.

Now that means are available for selecting a fraction of single polio particles by velocity-banding in a density gradient and for verifying by appropriate quantitative procedures for electron microscopy that they are indeed monodisperse (15) the kinetics of single particle reactions can be examined, perhaps for the first time. This is essential if the inactivation kinetics of virus in the complex environment of polluted water are to be analyzed and understood.

The reaction rates encountered here are very fast. Special apparatus has been employed for accurate determination of survival titers and reaction times as short as two seconds and all virus suspensions have been monitored by electron microscopy to insure a high degree of dispersion at the time of bromine contact.

Materials and Methods

Preparation, Purification and Plaquing of poliovirus: Poliovirus, type I, Mahoney strain, was obtained from Dr. Gerald Berg, U. S. Environmental Protection Agency, Cincinnati, Ohio, and was serially passaged in Human Epidermoid Carcinoma cells, HEp-2. The cells were grown in medium 199 containing 0.105% $NaHCO_3$ and 5% fetal calf serum (FCS). After the cells reached confluency they were maintained on the same medium with 2% FCS.

Stocks of virus were produced in HEP-2 cells at 37°C by infection at a multiplicity of 10-20 PFU/cell under a maintainence medium of 199 + 0.105% $NaHCO_3$ and 2% FCS. After 18-24 h when the CPE was 100%,

the cells were frozen and thawed 3 times and the cell debris removed by low-speed centrifugation (~800G) for 10 min. The supernatant fluids had titers of 4×10^{10} PFU/ml and were kept frozen at -70°C.

Purified virus was produced in the same cells grown for 3 days in 32-ounce prescription bottles inoculated with the above stock virus in Dulbecco's PBS containing 12.5 mM $MgCl_2$, at a multiplicity of ~1000 PFU/cell. The virus was allowed to adsorb for 1 h at 37°C, then 40 ml of maintainence medium containing 12.5 mM $MgCl_2$ was added to each bottle and the cells were further incubated at 37°C for 11 h. The cells were chilled to 4°C and the supernatant fluid was decanted. The monolayers were washed 2 times with PBS and the cells of each bottle were scraped into 10 ml of PBS and pelleted at 250 G for 10 min. Cells remaining in the supernatant fluid were pelleted similarly and pooled with the scraped cells and pelleted again. The pellet of cells was resuspended in 6 ml of PBS not containing Mg^{++} or Ca^{++}. Four ml of Freon 113 were added and the cells were homogenized at one-half speed in a Sorvall Omni-Mixer for 2 minutes. The phases were separated at 800G for 10 minutes and the upper aqueous phase was removed and held in an ice bath.

The Freon phase was re-extracted with a further 6 ml of PBS and the phases again separated. This aqueous phase was pooled with the first, and the Freon phase again re-extracted with 6 ml of PBS. All three aqueous phases were combined and made to a volume of 20 ml. Ten ml of this extract was placed on each of two 10 → 30% w/w sucrose gradients made in 0.05 M phosphate buffer, pH 7.2. The gradients were centrifuged at 25,000 RPM in the Beckman SW27 rotor at 4°C for 2 hours and 15 minutes. Fractions of 2 ml each were collected from the tubes and examined by the kinetic attachment method (16) for the presence of the virus. Twenty fractions were obtained from each tube and the highest count of virus was most frequently found in fraction 14. All relevant fractions were pooled and the virus was stored at refrigerator temperature without any attempt to remove the sucrose.

Plaque assays of poliovirus were performed on 4 day old monolayers of HEp-2 cells in 1-ounce prescription bottles under an overlay of 1% Difco agar containing medium 199 plus 5% FCS, 0.210% $NaHCO_3$, 0.003% neutral red, and 5 mM $MgCl_2$. The plaques were read after 3 days incubation at 37°C.

Physical Assay of the Virus: Purified virus preparations, recovered from the sucrose gradient usually at 22% concentration, can be assayed directly by the kinetic attachment (KA) method previously described (16). A minimum dilution of 1/5 is required to reduce the sucrose concentration to a level at which it does not interfere with the process. If further dilution is required to reduce virion concentration for optimum numbers per electron microscope picture it is helpful to include 5% sucrose in the diluent. We have obtained the most uniform results in this way.

Water. Preparation of demand-free water, phosphate buffered to pH 7 and adjustment to proper bromine concentration have been described in a previous paper (15).

Apparatus for exposure of virus to bromine action and removal of samples for titration after 1 to 20 seconds of exposure has been described in the preceding paper on reovirus (17).

Experiments and Results

When the virus used in these experiments is extracted from the infected HEp2 cells with freon and velocity-banded in a sucrose gradient the particle content of several successive fractions from such a gradient is shown in Fig. 8. The concentration of particles calculated from counts from such pictures is plotted against radial position in Fig. 9. Many particles smaller than virus, probably ribosomes, can be seen in fractions 11 and 12 just above the virus peak. These were not counted. At no point below the main virus peak was there any secondary peak in the region where pairs, triplets, etc. would have been expected had they been present in substantial numbers. It appears from this that the freon extraction process has yielded a virus preparation with a very high proportion of single particles.

The strip pictures of Fig. 8 were taken at high particle concentration, presenting enough particles so the reader may judge their relative number and degree of purity. Other pictures made with more dilute preparations were used to determine the true degree of aggregation, free of the coincidence effect of one particle falling upon another by accident. Calculation of the number of such accidental pairs will be made in the discussion. They indicate that the starting virus for these experiments contains at least 95% single particles.

Poliovirus inactivation by bromine at 2°C and pH 7

Six experiments were made at 2°C, three of which are shown graphically in Fig. 10. All were characterized by an initial linear phase, constant decline in log PFU survivor ratio per unit time of exposure. None of these reactions showed any tendency toward delay during the first time interval which was 4 seconds. The slopes of all the reactions ranging from 0.6 to 22 μM bromine concentration have been plotted (Fig. 11) where it can be seen that increasing bromine concentration does not produce a linear increase in reaction rate. The increase becomes progressively less with increasing bromine concentration indicating a progressive decrease in efficiency.

In several inactivation experiments at bromine concentrations of 3.5 μM or greater, there has been a slight increase in the reaction rate after a substantial period of linearity (see the 22 μM line of Fig. 10). We have not been able to produce this effect regularly. Slopes for Fig. 11 were taken from the linear part of each survival graph.

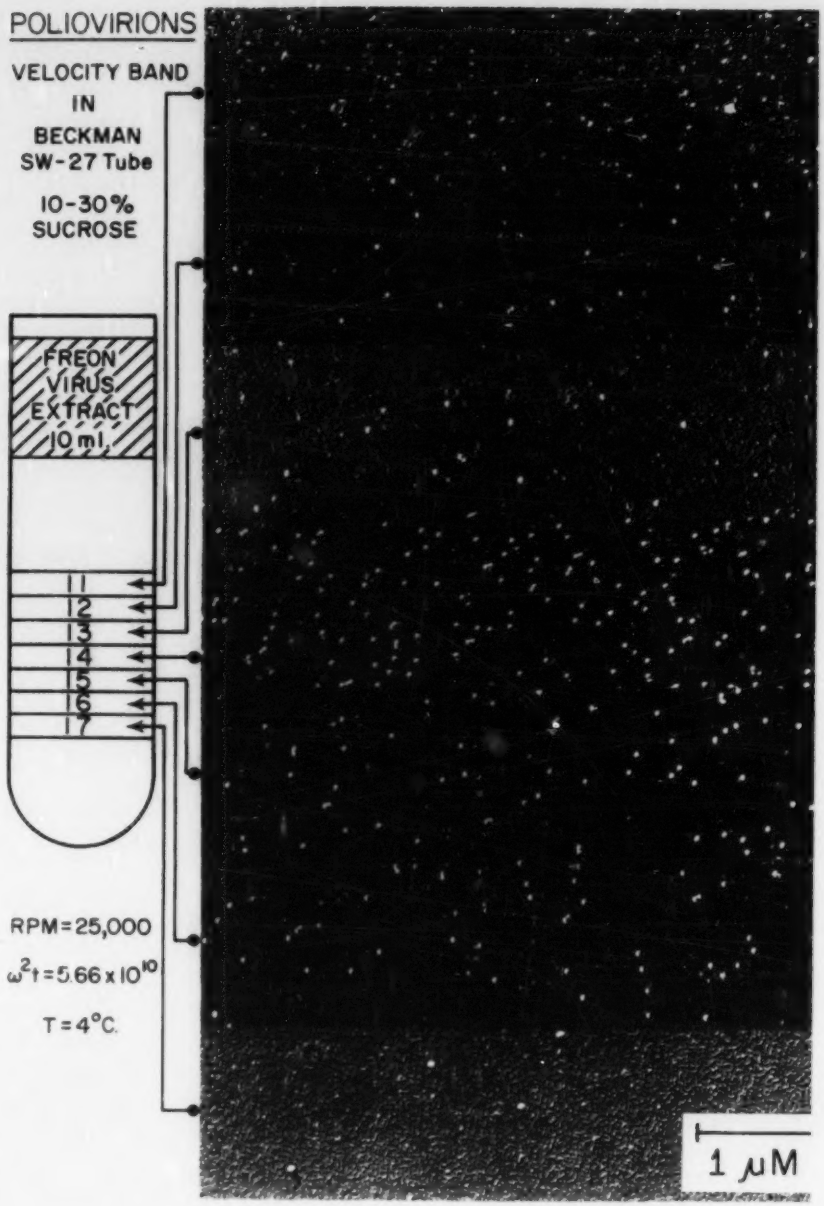

Fig. 8

Polio virus, velocity banded in a sucrose density gradient after freon extraction from infected HEp 2 cells.

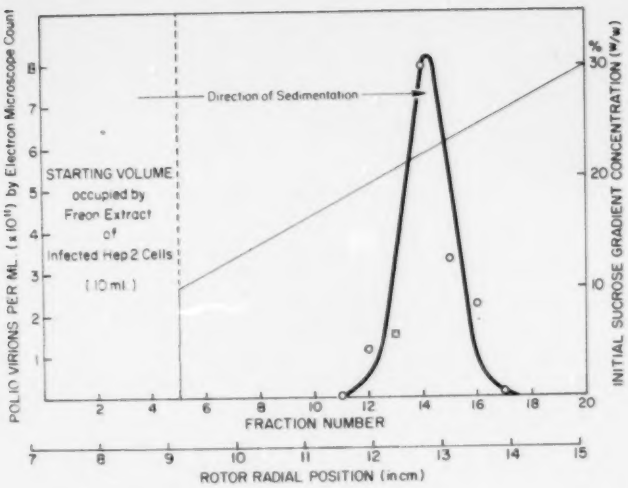

Fig. 9

Graphical display of polio virus particle count by electron microscopy, taken from the fractions and pictures shown in Fig. 8.

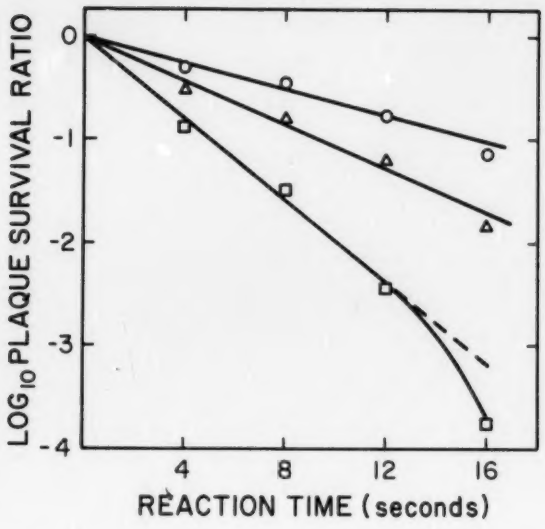

Fig. 10

Inactivation of poliovirus at 3 different concentrations of bromine at pH 7 and 2°C, top line 0.6 μM, middle line 2.2 μM and bottom line 22 μM.

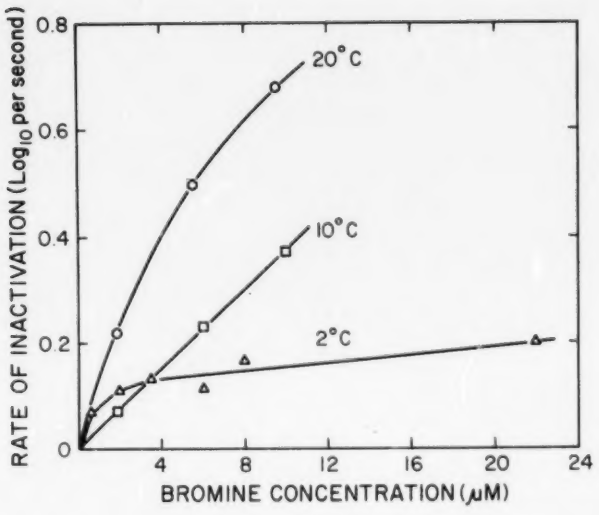

Fig. 11

Inactivation rates (Slopes in Logs/second taken from Figs. 10, 12, and 13) for poliovirus at pH 7 as function of temperature and bromine concentration.

Poliovirus inactivation by bromine at 10°C and pH 7

At 10°C the inactivation rate was constant at 1.9 μM bromine concentration (Fig. 12). There is no indication, in this experiment, that there is any time delay before inactivation begins. However, at higher bromine concentrations the linear part of the graph begins only after the first 4 second time interval (Fig. 12). It seems unlikely that the initial rate at the higher concentrations could be less than that of the straight line for 1.9 μM bromine so the lower curves have been drawn tangent to it at zero time. The reaction rates, slopes of the linear parts of the three kinetics experiments, are shown (Fig. 11) to be a linear function of bromine concentration with an intercept indicating zero reaction for zero bromine.

Poliovirus inactivation by bromine at 20°C and pH 7

At 20°C the inactivation of the virus proceeds faster at all bromine concentrations than it did at 10°C (Fig. 13). There is some indication in the graph for the 9.5 μM experiment that aggregation is showing its effect at survival levels below 10^{-3}. Reaction rates were determined from the straight part of each line.

Discussion

At 2°C the inactivation of poliovirus by bromine shows no evidence of delay even during the first 4 seconds under conditions leading to 99.9% plaque reduction in 16 seconds. The logarithm of plaque survival ratio decreases linearly with exposure time as might be expected from the electron microscope data that indicated over 95% of single particles in the preparation. At 10°C only the slowest reaction, the one at 1.9 μM bromine concentration, was strictly linear. The faster reactions at higher bromine concentrations showed a slight delay before linear decline rates were established (Fig. 12). Speculation in the past about such delay in the inactivation curve has often invoked aggregation as one of the possible explanations (11). Now, it is clear that the small amount of aggregation in this preparation can have no perceptible effect on the course of the reaction above the 10% survival level. Such a delay may be just the time required for penetration of the bromine to the critical point within the virus capsid.

Inactivation at 20°C was essentially first order over a 5 fold range of bromine concentration and it was substantially faster than that at 10° but the summary, Fig. 11, showing the dependence of reaction rates on both bromine concentrations and temperature, reveals an unexpected feature. The approach to zero rate at 2°C is quite different from that seen at the two higher temperatures. From bromine concentrations of 22 down 3.5 μM the rate falls slowly by only 36%. Beyond that point it plunges steeply to zero but not before crossing the 10°C line at 3.5 μM bromine level. This indicates that below 3.5 μM bromine concentration

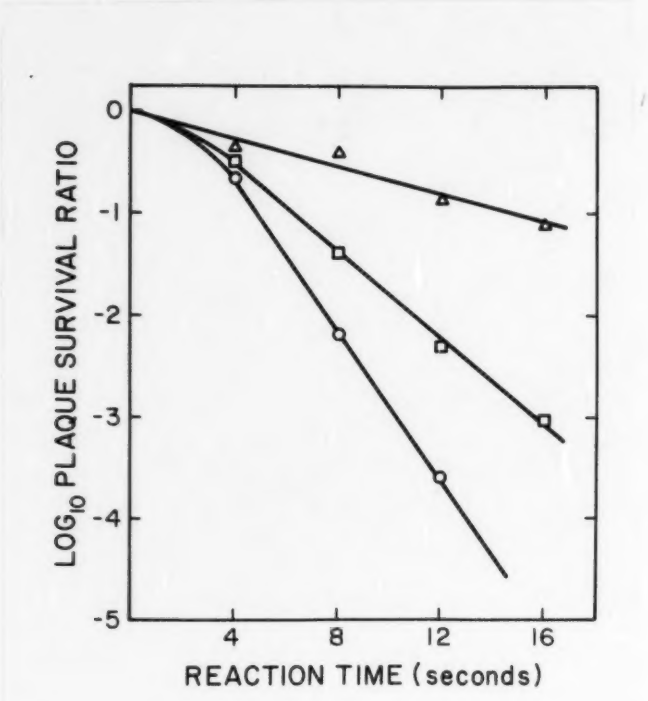

Fig. 12

Inactivation of poliovirus at 3 concentrations of bromine at pH 7 and 10°C, top line 1.9 μM, middle line 5.9 μM and bottom line 10 μM.

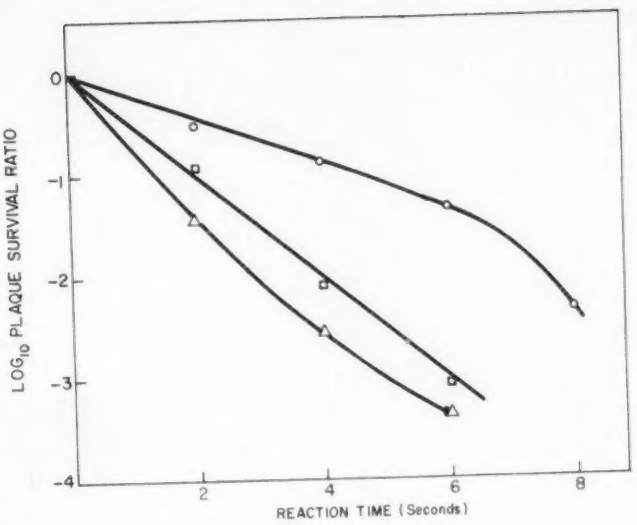

Fig. 13

Inactivation of poliovirus at 3 concentrations of bromine at pH 7 and 20°C, top line 1.9 µM, middle line 5.5 µM and bottom line 9.5 µM.

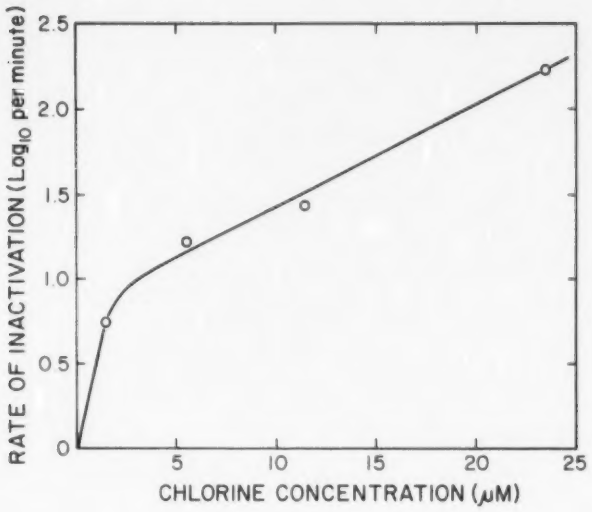

Fig. 14

Concentration dependence of the reaction rate of HOCl at $0^{o}C$ on poliovirus. These data have been taken with the permission of Dr. Weidenkopf from Table I of his publication (18).

the rate of poliovirus inactivation is greater at 2°C than at 10°C. At this time we have no mechanism to propose that would explain these facts. We are not aware of any significant error in measurement of bromine concentration even at the 1 μM level. However, there is evidence in the work of Weidenkopf (18) with chlorine on poliovirus, that at 0°C the inactivation rate depends on chlorine concentration in much the same way as we have seen at 2° with bromine. With Dr. Weidenkopf's kind permission we have plotted the data from Table I of his publication. Figure (14) shows a linear decrease in rate from 24 μM chlorine down to somewhat below 4 μM and then a rapid fall to zero. The break occurs at approximately the same molar concentration for both Br and Cl. Careful calculations made from the published graphs of Scarpino et al (19) with chlorine at 5°C definitely does not show this effect. It would appear to occur only at low temperatures and it may be related to changes in virion hydration near the transition point of water which is at 4°C.

A comparison of rates of bromine and chlorine inactivation of poliovirus seems appropriate at this point but it can be misleading. The data have been obtained here with essentially single virus particles and they indicate reaction rates much faster than those reported for chlorine. The data of Weidenkopf (Fig 14) shows at 10 μM chlorine (pH6) a decline in survival titer at 0° of 1.44 $\log_{10}$/minute. In Fig. 11 the rate for bromine at 2° was 6 times faster. From Scarpino et al (19) we have estimated that 10 μM chlorine (pH6 and 5°C) produces 0.8 $\log_{10}$/(min.) drop in plaque survival titer. Neither paper (18,19) provides any data on the temperature coefficient of the reaction so it is not possible to compare them directly but both indicate a reaction rate for chlorine at pH6 that is 1/5 to 1/10 of what we find for bromine. A more accurate comparison cannot be made until the effects of chlorine have been observed on essentially monodisperse virus preparations. We expect to report on this in a subsequent paper.

It is obvious that any method employed to collect on a plane surface, the particles suspended in a volume of fluid, must reveal some few aggregates by accidental coincidence even though there may have been none in the suspension. So, when none or a very few pairs exist in the suspension we have assumed that the chance of one particle falling upon another and producing an accidental pair in the electron micrograph is equal to the quotient of total area excluded by the particles divided by the total area of the surface photographed. The area excluded by a round particle of radius r already on the surface would be just $4\pi r^2$. The difference between the number of pairs so calculated and the number of pairs observed has been taken to be the true number of pairs present in the suspension and it has been so reported.

SUMMARY

A major part of this work has been the design and/or development of apparatus and techniques for producing, purifying, concentrating, selecting, assaying, and generally managing virus and its aggregation in water suspension and exposing such virus to the disinfecting effects of bromine. Physical assay methods had been devised for particle counting and aggregation analysis of both reo and the much smaller polio virus before this study was started but continuous improvements on both have been made as new needs have appeared. Sedimentation or spin-down counting, by EM, of reo preparations has shown the effect of small aggregates in producing curvature in the semi-log disinfection line (Chapter I). It showed, also that reo virus preparations must have well over 90% single particles in order to yield a straight line semi-log disinfection experiment extending 4 $\log_{10}$ units or more. Such preparations of single reo virions have been quite difficult to produce and even more difficult to maintain. They were, nevertheless, necessary to establish the rate of bromine action on single virus particles which has been done now (Chapter I). But this reaction is so fast that the classical methods that serve well with bacteria and chlorine are quite inadequate. It was necessary to design and construct a dynamic (flowing stream) experimental apparatus capable of exposing virus in water to bromine for accurately measured times as short as ½ second. This, too, has been completed and the results in Chapter I show its success with the sensitive single particle suspensions of reo virus. While these have been reduced over 4 $\log_{10}$ units in 1½ seconds, the fact that reo virus prepared in the usual way contains a few PFU that survive over ten times this exposure to bromine becomes a prominent fact of engineering significance. These survivors are too few to be found by electron microscopy but their size has been shown indirectly by sedimentation velocity separation in the zonal centrifuge.

Velocity analyses in the zonal and also in the swinging bucket rotors of the ultracentrifuge have provided significant data (Chapter I) on the partition of virus between singles, small aggregates of 2 to 10 particles and heavily aggregated clumps that may be combined with host or foreign material. Ordinarily the virologist has directed his attention to the first of these categories while suffering the consequences of the unwanted presence of the other two. Our centrifugation experiments have separated these three categories and they have been particularly revealing when applied to crude (unpurified) freeze-thaw lysates of infected cells. Such virus preparations are an approximation of polluted water and our early experiments show that over half of the PFU (sometimes over 90%) are either large virus clumps or virions attached to particles sedimenting much faster than single virions. Present particle count data by EM has not shown ratios (dispersed virus/aggregated virus) this great. At present it appears that the aggregated reo virus produces plaques more efficiently than do the single unencumbered virions.

Supporting data for the above summary statements about reo virus are in the first chapter of this report and they show the extraordinary value of reovirus as a model for this kind of work. It is sufficiently large to permit excellent correlations of physical data from the electron microscope with biological titration. While it is highly probable that the small enteric viruses will have different properties, as Chapter II will show for polio, experience gained with reo has often pointed the way to go into this more difficult area.

Polio virus results are the subject of Chapter II. The preparative methods described are qualitatively the same as those used for reo but the results differ in one prominent way. The fraction of single virions in velocity-banded freon-extracted preparations has been very high, usually over 90% of the total particle count by the KA method of electron microscopy. This surprising result has permitted a series of experiments with bromine at different concentrations and temperatures which are, in some respects far ahead of those with reo virus.

Polio virus, single particle suspensions, are more resistant to bromine than reo. At $2^{\circ}C$ in 3 μM bromine at pH 7 the ratio of disinfection rates is about 25:1. But in spite of this great difference in resistance, the bromine inactivation of poliovirus is still fast enough to require samples exposed as little as 4 seconds. The continuous flow apparatus is still necessary with polio virus. Inactivation data so far gathered with highly dispersed virus preparations now covers bromine concentrations from 0.6 to 22 μM and temperature from 2° to 20°. As a first approximation one would say that these data show all the inactivations are first order reactions. There are, however, two kinds of exceptions noted and this time they cannot be attributed to aggregation of the virions. The first of these was a slight lag in the starting phase noted only in two experiments at $10^{\circ}C$. It could be related to penetration time for bromine through the virus capsid. The second observed departure from expected classical behavior has appeared only at $2^{\circ}C$ but it has been established in many experiments and with different preparations of virus. It appears as a lack of proportionality between reaction rate and bromine concentration. Low concentrations (0.2 to 2.5 μM) are much more efficient in destroying polio virus at $2^{\circ}C$ than higher concentrations ranging up to 22 μM. This means that the Arrhenius plot is not straight at low bromine concentrations, a fact that usually indicates that the nature of the reaction is not the same in this region as it is at higher concentrations. We have no interpretation of this phenomenon at this time but only a suggestion that all virus particles are known to be hydrated and water has a transition in density at $4^{\circ}C$. The inactivation rate for reo virus may be dependent in some way upon virion hydration which could also have a transition in the same temperature region. Data taken at $10^{\circ}C$ and $20^{\circ}C$ do no show this inconsistency.

CONCLUSIONS

The general state of virus particles in water suspension is aggregated. Many viruses are produced as aggregates in infected cells and when we have examined suspensions of reovirus by zonal centrifugation or by quantitative electron microscopy we have found a substantial fraction (often over 50%) sedimenting much faster than single particles and in this fraction many aggregates can be seen by EM.

When aggregates are present in a reo virus suspension containing bromine, the disinfection rate of the mixture proceeds at a constantly decreasing rate which appears to be determined by the frequency of the different aggregate sizes in the mixture.

When aggregates are removed from the above mixture by taking advantage of their difference in sedimentation velocity, the rate of disinfection of the remainder by bromine becomes essentially a straight line. This is the basic reaction rate for single virions and it is the only reliable basis for comparing the resistance of one virus with another with different disinfectants under different conditions.

Polio virus suspensions of single particles are about 25 X more resistant to bromine than similar suspensions of reo virus, and there are some anomalies too. In a limited range of temperature and bromine concentration there is a slight delay in the onset of the disinfection process. Such delays have been seen by others but always with what was presumed to be aggregated virus. This delay must now be attributed to something else, possibly penetration of the virus capsid by the bromine. Polio virus disinfection rates are proportional to bromine concentration at 10^{o} and at 20^{o}C but at 2^{o}C they are not. Reasons for this are not apparent. This leads to a linear Arrhenius plot only at about 10 µM bromine concentration but not at other levels.

When working with bromine and suspensions of single virions it is necessary to make very short time exposures. Even with polio virus and 3 µM bromine they must be as short as 5 seconds and with reo virus, ½ second or less. The continuous flow or "Flash Flood" apparatus that we have built has met this need and it promises to be of broad general value in such work.

Preparation and storage of virus with a given degree of aggregation continues to be difficult, still it is essential to this work for who can experiment with aggregated virus unless he can produce it as needed for the experiment. The observed stability of both plaque titer and dispersion of both reo and polio virus in high concentrations of sucrose promise to be a finding of great practical significance for it provides a starting point for continued experimentation on virus aggregation.

RECOMMENDATIONS FOR RESEARCH THAT SHOULD BE DONE

1. A. Continue Br and Cl inactivation experiments with single reo and polio virus particles until relative and absolute inactivation rates have been established under a sufficient variety of conditions (pH, Temp. etc.) so that the accumulated data can be taken as a firm front of reference by other virus workers and also for our work with aggregated viruses as well.
   B. Examine other enteric viruses in the same way.

2. Examine the curvature that we have seen in bromine inactivation experiments with slightly aggregated reovirus. Is this survival pattern produced by multiplicity reactivation in surviving small groups giving a Poisson-like curve of survival or is it produced by large groups that have survived to various degrees because of protection from penetration of bromine into the group. Zonal centrifugation of aggregated virus preparations prior to bromine treatment could provide data to answer this question.

3. Search for means of inducing aggregation of virus under controlled conditions and keeping such preparations and making PFU titrations of them.

4. Look for conditions under which an aggregated virus preparation will become dispersed. (We have not yet seen this happen).

5. Try to find out how virus is preserved in a dispersed and active condition by sucrose in high concentration.

6. Examine the reported "stabilizing effect" of $MgCl_2$ on the PFU titer of several enteric viruses. This phenomenon may be related to aggregation and it may be observable directly by electron microscope.

7. Examine the aggregating effect we have seen produced on reo virus by Bentonite. This may occur in water treatment processes now in use and it may influence the survival of virus in these processes.

REFERENCES

1. Sharp, D. G., R. Floyd and J. D. Johnson (1975). Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine. Applied Microbiology 29:94-101.

2. Sharp, D. G. and P. M. McGuire (1970). Spectrum of Physical Properties Among the Virions of a Whole Population of Vaccinia Virus Particles. J. Virology 5:275-281.

3. Anderson, N. G. (1966). The Development of Zonal Centrifuges. National Cancer Institute Monograph 21. U. S. Government Printing Office, Washington, D. C.

4. Taylor, D. G. and J. D. Johnson (1974). In A. J. Rubin (ed) Chemistry of Water Supply Treatment and Distribution. pg. 369. Ann Arbor Science Publishers, Ann Arbor, Mich.

5. Kim, K. S. and D. G. Sharp (1966). Electron microscopic observations on the nature of vaccinia virus particle aggregation. J. Immunology 97:197-202.

6. Geister, R. G. and D. H. A. Peters (1965). Quantitative use of the electron microscope in virus research. Method of analyzing and predicting biologic titers of aggregated virus suspensions with a new law of aggregation. Laboratory Investigation 14:864-874.

7. Sharp, D. G., R. Floyd and J. D. Johnson (1975). The inactivation by bromine of single poliovirus particles in water. Submitted for publication with the manuscript.

8. Scarpino, P. V., Gerald Berg, S. L. Chang, Daniel Dahling and Merrilee Lucas (1972). A comparative study of the inactivation of viruses in water by chlorine. Water Research 6:959-965.

9. Weidenkopf, S. J. (1958) Inactivation of Type 1 poliomyelitis virus with chlorine. Virology 5:56-67.

10. Salk, J. E., and J. B. Gori (1960). A review of theoretical, experimental and practical considerations in the use of formaldehyde for inactivation of Poliovirus. Ann. N. Y. Acad. Sci. 83:609-637.

11. Berg, G. S., S. L. Chang and E. K. Harris (1964). Devitalization of Microorganisms by Iodine I. Dynamics of the devitalization of Enteroviruses by elemental Iodine. Virology 22:469-481.

12. Clark, R. M. (1968). A Mathamatical Model of the Kinetics of Viral Devitalization. Mathematical Biostatistics 2:413-423.

13. Sharp, D. G. (1967). Electron Microscopy and Viral Particle Function in Transmission of viruses by the Water Route. Ed. Gerald Berg, Interscience Publishers, N. Y. pp. 193-217.

14. Davis, D. B., Dulbecco, R., Eisen, H. N., Ginsberg, H. S. and Wood, W. B. (1973). Microbiology, Harper Rowe, N. Y. pg. 1288.

15. Sharp, D. G., R. Floyd, and J. D. Johnson (1975). Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine. Applied Microbiology 29:94-101.

16. Sharp, D. G. (1974). Physical Assay of Purified Viruses, particularly the small ones. In C. J. Arceneaux (ed) Proc. 32nd. Annual Meeting of the Electron Microscopy Society of America. Claitor's Publishing Div. Baton Rouge, La. pp. 264-265.

17. Sharp, D. G., R. Floyd and J. D. Johnson (1974). Inactivation of Reovirus by Bromine in Water I. The Initial Rapid Phase. Submitted for Publication with this paper.

18. Weidenkopf, S. J. (1958). Inactivation of Type I Poliomyelitis Virus with Chlorine. Virology 5:56-67.

19. Scarpino, P. V., Gerald Berg, S. L. Chang, Daniel Dahling and Merrilee Lucas (1972). A comparative study of the inactivation of viruses in water by chlorine. Water Research 6:959-965.

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